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(54) Title: ANTICOAGULANT PROTEINS

(57) Abstract

Mutant recombinant TAP proteins which inhibit Factor Xa and thrombus formation. These proteins are site-directed mutants of wild type TAP, a Factor Xa inhibitor present in *Ornithodoros moubata* tick extract.

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TITLE OF THE INVENTION

ANTICOAGULANT PROTEINS

BACKGROUND OF THE INVENTION

The role of blood coagulation is to provide an insoluble fibrin matrix for consolidation and stabilization of a haemostatic plug. Formation of a cross-linked fibrin clot results from a series of biochemical interactions involving a range of well-characterized plasma proteins.

The interactions are divided into what are termed the "intrinsic pathway", in which all the substances necessary for fibrin formation are present in precursor form in circulating plasma, and the extrinsic pathway in which thromboplastin, derived from tissues, bypasses several steps in the process and accelerates clot formation. The two pathways are highly interdependent and Factor VII, Factor IX and Factor X are mutually activated (J.C. Giddings "Molecular Genetics and Immunoanalysis in Blood Coagulation" Ellis Horwood Ltd., Chichester, England (1988), p. 17).

The role of Factor X in the coagulation cascade has been reviewed by Zur *et al.* "Tissue factor pathways of blood coagulation", "Haemostasis and Thrombosis", 1st Edition (Bloom *et al.*, Eds) Churchill Livingstone, Edinburgh, pp. 124-139 (1981); Jackson, "The biochemistry of prothrombin activation", "Haemostasis and Thrombosis", 2nd edition (Bloom *et al.*, Eds) Churchill Livingstone, Edinburgh, pp. 165-191 (1987) and Steinberg *et al.* "The activation of Factor X" "Haemostasis and Thrombosis", First Edition (Colman *et al.*, Eds) Lippincott, Philadelphia, pp. 91-111 (1982).

Human Factor X circulates in plasma as a two-chain glycoprotein with a molecular weight of about 67000 estimated by gel electrophoresis in the presence of sodium dodecylsulphate (Di Scipio *et al.*, "A comparison of human prothrombin, Factor IX (Christmas factor), Factor X (Stuart factor) and protein S", *Biochemistry*, vol. 16, pp. 698-706). Slightly lower Mr values of about 59000 are obtained by sedimentation equilibrium analysis. The normal plasma concentration is

about 7-10 mg per liter and the protein contains about 15% carbohydrate.

The heavy chain of Factor X, Mr=42000 demonstrates a high degree of homology with prethrombin 2 and contains the active site serine. It is covalently linked to the light chain, Mr=17000, by disulphide bridges. The light chain contains the γ-carboxylated glutamic acid residues and shows significant homology with prothrombin fragment 1. Activation of Factor X by complexes of Factor IXa and Factor VIIIa or by tissue factor/Factor VIIa involves at least one peptide cleavage. The principal mechanism releases a small activation peptide from the heavy chain of the molecule by hydrolysis of an arginine-isoleucine bond. The active product formed in this way is termed α-Factor Xa. This is modified further by cleavage of an arginine-glycine bond near the carboxy terminus to form β-Factor Xa. Both α-Factor Xa and β-Factor Xa have the same coagulation activity.

The light chain of Factor X is unaffected by the activation process and remains linked to the heavy chain of the molecule by disulphide bridges. In this way the active substance retains the γ-carboxyglutamic acid domains necessary for calcium-mediated attachment to phospholipid micelles or cellular surfaces. Fundamentally, therefore, in contrast to thrombin, Factor Xa remains associated with phospholipid and platelet membranes.

European Publication 419099 describes a Factor Xa inhibitor derived from *Ornithodoros moubata* tick extract (TAP), a method for recombinantly producing TAP in yeast, and several conservatively mutated forms of TAP which retain anticoagulant properties.

Vermulen *et al.*, *Int. J. Biochem.*, Vol. 20, No. 6, pp. 621-31 (1988), describes the relative protease inhibition activities of tick toxins isolated from *R. evertsi evertsi*, *B. decoloratus*, *B. microplus*, and *H. truncatum*. They were found to be fast-binding or slow-binding inhibitors of trypsin and chymotrypsin.

Willadsen and Riding, *Biochem J.*, Vol. 189, pp. 295-303 (1980), describe activity of a proteolytic-enzyme inhibitor from the

ectoparasitic tick *Boophilus microplus*, and the effect of the inhibitor on blood-coagulation parameters.

Prior studies identified and partially purified inhibitors of Factor Xa Markwardt, F. *et al.* (1958) *Naturwissenschaften 45*, pp. 398-399 and Markwardt, F. *et al.* (1961) *Naturwissenschaften 48* p. 433 and thrombin Hawkins, R. *et al.* (1967) *Proceedings of the Royal Society* p. 70 in tick saliva. It has been suggested that saliva contains an inhibitor of Factor IXa (Hawkins). (Hellmann, K. and Hawkins (1967) *Thromb. Diath. Haemorrh. 18* pp. 617-625) Ribeiro *et al.* reported that tick saliva blocks clotting by inhibiting the intrinsic pathway (Ribeiro, J. (1985) *J. Exp. Med. 161* pp. 332-344) but they did not identify the site of inhibition. This study also demonstrated for the first time antiplatelet activity which blocked platelet aggregation induced by ADP, collagen, or platelet activating factor (Ribeiro, J. (1985) *J. Exp. Med. 161* pp. 332-344). No attempt was made to purify any of these factors to enable an analysis of their structures or mechanism of action.

The *S. cerevisiae* α-mating factor pre-pro leader sequence has been utilized in the expression of heterologous genes as secreted products in yeast (Brake *et al.*, *Proc. Natl. Acad. Sci. USA* vol. 81, pp. 4642-4646 (1984); Miyajima *et al.*, *Gene* vol. 37, pp. 155-161 (1985); Vlasuk *et al.*, *J. Biol. Chem.* vol. 261, pp. 4789-4796 (1986); Schultz *et al.*, *Gene* vol. 54, pp. 113-123 (1987); Schultz *et al.*, *Gene* vol. 61, pp. 123-133 (1987); Bayne *et al.*, *Gene*, vol. 66 pp. 235-244 (1988); and Laison *et al.*, *Biotechnology*, vol. 6, pp. 72-77 (1988)). Proteins produced as fusion products are proteolytically processed by the Lys-Arg-cleaving endopeptidase (KEX2) encoded by the KEX2 gene and products are secreted into the culture medium. The KEX2 cleaves on the C-terminal side of Lys-Arg residues which are present between the ppL sequence and the heterologous gene.

SUMMARY OF THE INVENTION

Proteins of the invention are more potent inhibitors of Factor Xa than wild type TAP. The mutant rTAP proteins dissociate from the mutant rTAP:Factor Xa complex at a significantly slower rate

than does wild type TAP from the wild type TAP:Factor Xa complex.

The present invention includes mutant recombinant TAP proteins and variants which specifically inhibit coagulation Factor Xa. The proteins are low molecular weight polypeptides having about 60 amino acids which are highly specific for Factor Xa. They do not significantly inhibit (less than 25% at 1 μM) human Factor VIIa, bovine trypsin, bovine chymotrypsin, human thrombin, human high molecular weight urokinase, human plasmin, human tissue plasminogen activator, porcine pancreatic elastase, human activated protein C, human plasma kallikrein and bovine tissue kallikrein.

The mutant recombinant TAP proteins of the present invention contain amino acid substitutions at positions 1 and 10 of the wild type TAP sequence. The proteins of the present invention can contain either a single mutation at amino acid 1, a single mutation at amino acid 10, or mutations at both amino acid 1 and amino acid 10. Mutants of the present invention containing mutations at both positions are called double mutants. The present invention also encompasses mutant recombinant TAP proteins containing mutations in addition to mutations at positions 1 and 10 (e.g. triple mutants containing mutations at positions 1, 10, and another amino acid), provided that the additional mutations are conservative or do not substantially affect the Factor Xa inhibitory properties of the double mutant protein or the rate of dissociation between the double mutant protein and Factor Xa.

The present invention also includes compositions comprising a protein of the invention which inhibits Factor Xa and prevents coagulation in patients, and methods for inhibiting blood coagulation comprising administering to the patient therapeutically effective amounts of the compositions.

The present invention also includes methods for recombinantly producing the proteins in yeast cells and recovering the expressed proteins, and vectors for transforming the yeast cells.

DESCRIPTION OF THE FIGURE

Figure 1 represents double mutant recombinant TAP Y1W/D10R .

DETAILED DESCRIPTION OF THE INVENTION

Standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C).Corresponding nucleotides are, for example, deoxyadenosine-5'-triphosphate (dATP). Amino acids are shown either by three letter or one letter abbreviations

Wild type TAP is described in European Publication 419099. The proteins of the present invention are prepared using site directed mutagenesis.

Single mutant rTAP proteins include such proteins having single amino acid substitutions at position 1 (where the wild type TAP amino acid is tyrosine) or position 10 (where the wild type TAP amino acid is aspartate). Amino acid 1 is substituted with a highly hydrophobic amino acid, preferably one having an aromatic side chain. More preferred amino acids at position 1 are selected from the group of amino acids consisting of tryptophan, phenylalanine, or β-naphthyl-alanine. Even more preferably, the amino acid is tryptophan (this more preferred single mutant rTAP protein is "Y1W" or "Trp^1-TAP"). Amino acid 10 is substituted with a highly hydrophilic amino acid, preferably basic and positively charged and having a polar side chain. Preferred amino acids at amino acid position 10 are selected from the group of amino acids consisting of arginine, lysine, and histidine. More preferably, the amino acid is arginine (this more preferred single mutant rTAP protein is "D10R" or "Arg^{10}-TAP").

Double mutant rTAP proteins of the present invention have two specific amino acid substitution mutations. Double mutant TAP proteins of the present invention are more preferred than single mutant TAP proteins. One mutation is at amino acid 1 (where the wild type TAP amino acid is tyrosine) and the other is at amino acid 10 (where the wild type TAP amino acid is aspartate). Amino acid 1 is substituted

with a highly hydrophobic amino acid having an aromatic side chain. Preferred amino acids at position 1 are selected from the group of amino acids consisting of tryptophan, phenylalanine, or β-naphthylalanine. More preferably, the amino acid is tryptophan, which is more hydrophobic than tyrosine. Amino acid 10 is substituted with a hydrophilic amino acid, preferably basic and positively charged and having a polar side chain. Preferred amino acids at amino acid position 10 are selected from the group of amino acids consisting of arginine, lysine, and histidine. More preferably, the amino acid is arginine. The most preferred double mutant protein (referenced hereinafter as "Y1W/D10R", or "$Trp^1 Arg^{10}$-TAP") has a substitution combination of amino acid 1 as tryptophan (replacing wild type tyrosine) and amino acid 10 as arginine (replacing wild type aspartate).

Also considered as within the scope of the present invention are recombinant TAP proteins containing mutations in addition to mutations at positions 1 and 10 (e.g. triple mutants containing mutations at positions 1, 10, and another amino acid), provided that the additional mutations are conservative or do not substantially affect the Factor Xa inhibitory properties of the double mutant protein or the rate of dissociation between the double mutant protein and Factor Xa. European Publication 419099 is illustrative of mutation strategies which would be expected to provide sequences that retain activity. Recombinant proteins having mutations of wild type TAP at amino acid positions 9, 25, 29, and 53, replacing arginine with asparagine, were shown to inhibit Factor Xa. It is therefore expected that such additional mutations of the single mutants (at amino acid positions 1 or 10) and double mutants (at amino acid positions 1 and 10) described above would retain potency.

Both single mutant TAP proteins and double mutant TAP proteins of the invention are more effective Factor Xa inhibitors than wild type TAP. Regarding human Factor Xa inhibitory activities, the proteins of the present invention have a significantly slower dissociation rate than the dissociation rate corresponding to the wild type TAP. The most preferred protein, Y1W/D10R, is 14 times slower.

Generally, proteins of the present invention can be prepared according to the recombinant procedures described below.

In the instance where the protein includes commercially available amino acid β-naphthylalanine at amino acid position 1, the protein can be prepared by first preparing the minus 1 mutant of wild type TAP (amino acids 2-60 of wild type TAP) or the minus 1 mutant of TAP having an amino acid substitution for amino acid 10 of wild type TAP, as described above (amino acids 2-60 of a mutant TAP having arginine, lysine, or histidine at position 10). Thereafter, an activated, protected β-naphthylalanine derivative can be attached to amino acid 2 under proper protection and pH conditions.

Alternatively, the first prepared minus 1 mutant can also have an amino acid replacement for the amino acid at position 7 of wild type TAP. In this alternative, wild type TAP lysine (Lys^7-TAP, or wild type TAP) is replaced with arginine (Arg^7-TAP). Amino acid 10 may optionally be changed as described above. Thereafter, an activated, protected β-naphthylalanine derivative can be more conveniently attached to amino acid 2.

Standard protection procedures, activation procedures, coupling procedures, and deprotection procedures necessary for attaching an amino acid such as β-naphthylalanine to amino acid 2 in minus 1 mutants of rTAP would be obvious to a person skilled in the art in view various peptide synthesis publications such as Merrifield, J. Am. Chem. Soc., 85, 2149 (1964), Rivier *et al.*, United States Patent 4,244,946 (1982), and Stewart and Young, "Solid Phase Peptide Synthesis", Pierce Chemical Company, Rockford, IL (1984).

<u>Proteins</u>

Proteins of the invention include variations on the disclosed protein sequence or sequences which conserve the activity of the disclosed sequence or sequences, including fragments or subunits, naturally occurring mutations, allelic variants, randomly generated artificial mutants, and microheterogeneous forms.

Fragments or subunits refers to any portion of the sequence which contains fewer amino acids than the complete protein, e.g. partial sequences excluding portions at the N- and/or C-termini of the complete protein.

Microheterogeneous forms, as used herein, refers to a single gene product, that is a protein produced from a single gene unit of DNA, which is structurally modified following translation. These structural modifications, however, do not result in any significant alterations of the activity of the protein. The modifications may take place either *in vivo* or during the isolation and purification process. *In vivo* modification may result in, but is not limited to, acetylation at the N-terminus, proteolysis, glycosylation or phosphorylation. Proteolysis may include exoproteolysis, wherein one or more terminal amino acids are sequentially, enzymatically cleaved to produce microheterogeneous forms having fewer amino acids than the original gene product. Proteolysis may also include endoproteolytic modification that results from the action of endoproteases that cleave the peptide at specific sequence locations. Similar modifications, especially proteolytic modifications, can occur during the purification process which may result in the production of microheterogeneous forms.

Also included are hybrid proteins, such as fusion proteins or proteins resulting from the expression of multiple genes within the expression vector, and may include a polypeptide having the specific activity of a disclosed protein linked by peptide bonds to a second polypeptide.

It will be understood that other variants of proteins of the present invention which conserve activity are included, especially variants that differ from the isolated proteins only by conservative amino acid substitution. Conservative amino acid substitutions are defined as "sets" in Table 1 of Taylor, W.R., *J. Mol. Biol.*, Vol. 188, p. 233 (1986).

Also within the present invention are bifunctional proteins having a polypeptide sequence comprising the mutant rTAP protein and included therein the tripeptide sequence arginine-glycine-aspartic acid

(RGD). The presence of the RGD tripeptide is achieved, for example, by inserting glycine between amino acid positions 9 and 10, or between amino acid positions 53 and 54.

The bifunctional molecules are particularly suited for targeting proteins of the present invention to activated platelets where there is an assembled prothrombinase complex. The prothrombinase complex, composed of Factor Xa, Factor Va, an appropriate phospholipid surface and prothrombin, is responsible for the Factor Xa conversion of prothrombin to thrombin *in vivo*. The phospholipid component of this complex can be supplied by any cellular surface. However, it has been demonstrated that upon activation, platelets vesicularize into microparticles which expose Factor Va binding sites. These activated platelet micro particles are extremely active in supporting the prothrombinase complex. Insertion of an RGD sequence in an appropriate location of a non-adhesive protein creates a protein capable of binding to integrins which recognize this peptide sequence such as the glycoprotein IIb/IIIa protein (Maeda, T. *et al.* (1989), *J. Biol. Chem.* 264, 15165-15168). Upon activation, the platelet IIb/IIIa complex becomes exposed and is critical in the subsequent fibrinogen-mediated aggregation. Insertion of an RGD sequence in an appropriate region of mutant rTAP of the present invention by, for example, site-specific mutagenesis, creates a bifunctional molecule targeted to activated platelets where the prothrombinase complex is assembled.

Recombinant DNA Technology

Recombinant DNA technology may be used to produce proteins of the invention. This technology allows segments of genetic information, DNA, from different cells, and usually from different organisms, to be joined end-to-end outside the organisms from which the DNA was obtained and to incorporate this hybrid DNA into a cell that will allow the production of the protein for which the original DNA encodes. Genetic information, DNA or mRNA, is isolated and incorporated into an appropriate cloning vector, and transduced into an appropriate host cell.

Cloning vectors useful for this technology include a DNA sequence which accommodates specific experimental foreign DNA. The vectors are introduced into host cells that can exist in a stable manner and express the protein dictated by the experimental DNA. Cloning vectors may include plasmids, bacteriophage, viruses and cosmids.

Expression vectors are DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. These vectors can express either procaryotic or eucaryotic genes in a variety of cells such as bacteria, yeast, insect and mammalian cells.

Proteins may also be expressed in a number of virus systems. A suitably constructed expression vector contains an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. Promoters are DNA sequences that direct RNA polymerase to bind to DNA and initiate RNA synthesis; strong promoters cause such initiation at high frequency. Expression vectors may include, but are not limited to cloning vectors, modified cloning vectors and specifically designed plasmids or viruses.

<u>Expression Systems</u>

Procaryotes most frequently are represented by various strains of *E. coli*. Other microbial strains may be used, such as bacilli, e.g. *Bacillus subtilis*, various species of Pseudomonas, or other bacterial strains. In such procaryotic systems, plasmid vectors which contain replication sites and control sequences derived from a species compatible with the host are used. For example, *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species by Bolivar *et al.*, *Gene* (1977) *2*:95. Commonly used procaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the β-lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.*, *Nature* (1977) <u>198</u>:1056) and the

tryptophan (Trp) promoter system (Goeddel *et al.*, *Nucleic Acids Res.* (1980) *8*:4057) and the lambda-derived P_L promoter and N-gene ribosome binding site (Shimatake *et al.*, *Nature* (1981) *292*:128). However, any available promoter system compatible with procaryotes can be used.

Expression systems useful in eucaryotic systems of the invention comprise promoters derived from appropriate eucaryotic genes. A class of promoters useful in yeast, for example, include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.* (1980) *255*:2073). Other promoters include those from the enolase gene (Holland, M.J., *et al.*, *J. Biol. Chem.* (1981) *256*:1385) or the Leu2 gene obtained from YEp13 (Broach, J., *et al.*, *Gene* (1978) 8:121).

Mutant rTAP proteins of the present invention are preferably expressed in eukaryotic systems such as transformed yeast cell expression systems, more preferably transformed *Saccharomyces cerevisiae* strain DMY6. More preferably, the transformed yeast cell expression system is DMY6(TAP3).

Oligonucleotide Primers

Oligonucleotide primers are prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid of defined length. The primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods, described respectively in Narang, S.A., *et al. Meth. Enzymol.*, *68*, 90 (1979) and Brown, E.L. *et al.*, *Meth. Enzymol. 68*, 109 (1979), or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage *et al.*, *Tetrahedron Letters* (1981), *22*: 1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

Polymerase Chain Reaction Amplification

Large amounts of DNA coding for mutant rTAP proteins may be obtained using polymerase chain reaction (PCR) amplification techniques as described in Mullis *et al.*, U.S. Patent No. 4,800,159. The extension product of one primer, when hybridized to another primer, becomes a template for the production of the nucleic acid sequence.

The nucleic acid sequence strands are heated until they separate, in the presence of oligonucleotide primers that bind to their complementary strand at a particular site of the template. The primer template complexes act as substrate for DNA polymerase which, in performing its replication function, extends the primers. The region in common with both primer extensions, upon denaturation, serves as template for a repeated primer extension. This process is continued with a series of heating and cooling cycles, heating to separate strands, and cooling to reanneal and extend the sequences. More and more copies of the strands are generated as the cycle is repeated. Through amplification, the coding domain and any additional primer-encoded information such as restriction sites or translation signals (signal sequences, start codons and/or stop codons) is obtained.

Mutagenic Methods

PCR Mutagenesis

The use of polymerase chain reaction catalyzed by *Taq* DNA polymerase for site-specific mutagenesis is described in Kadowaki *et al.*, *Gene* 76 (1989) 161-166. Kadowaka *et al.* describe introduction of mismatches into the oligos used to prime polymerase chain reactions (Saiki, *et al.*, *Science* 230 (1985) 1350-1354 and Saiki *et al.*, *Science* 239 (1988) 487-491) in order to adopt the method to site-directed mutagenesis.

PCR Mutagenesis - Near-terminal location mutations

Mutations occurring near the ends of the coding sequence were modified through polymerase chain reaction (PCR) amplification. By adding oligomer primers (complementary to the ends of the DNA to be amplified) to the TAP-encoding DNA and heating the reaction mixture, the DNA strands separate and permit annealling of the primers to the template. The primers bind opposite strands and, upon reaction with Taq polymerase (Cetus Perkin-Elmer) in a number of cycles of extension, strand separation and reannealling, the region between the primers is replicated. If one or more of the primers includes a mismatched region, that mutation is incorporated into the reaction product. TAP mutants were created through this amplification using the appropriate codon changes within the mutagenic primer.

PCR Mutagenesis - Non-terminal location mutations

A modification of this approach can be used for conservative mutations desired at non-terminal locations, occurring near unique Taq1 or Xho1 restriction sites. Non-terminal location mutations, for example, can be generated at residue positions 9 and 23, resulting in replacement of arginine with asparagine. Primers are prepared to amplify the TAP coding sequences and incorporate the mutations as before. The primers extend far enough to include restriction site sequences present in the TAP gene. Another set of primers is used to amplify remaining sequences of gene and the same restriction site. The resulting fragments are digested with that enzyme and ligated, reforming the TAP gene complete with the mutation.

Kunkel Mutagenesis

Kunkel *Proc. Natl. Acad. Sci. USA* Vol. 82 (1985) 488-492, describes site-specific mutagenesis which takes advantage of a strong biological selection against the original, unaltered genotype. Site-directed mutagenesis of amino acid 10 can be used to prepare the mutant rTAP of the present invention. By using a relatively normal uracil-containing DNA template, prepared by standard procedures

(Sagher and Strauss *Biochemistry* 22 (1983) 4518-4526) after growth on an *Escherichia coli dut⁻ ung⁻* strain (Tye *et al. Proc. Natl. Acad. Sci. USA* 75 (1978) 233-237), site-specific mutagenesis procedures are used to produce mutations at high efficiencies without selection and in a few hours.

Mutants can be prepared using a modification of the Kunkel method in which single strand p276-2E plasmid DNA was annealed with a complementary primer containing the desired mutagenic sequences. In European Publication 419099, the Kunkel method was specifically employed to obtain a wild type TAP mutation at residue position 27 which results in replacement of arginine by asparagine. This complex was extended by DNA polymerase, covalently linked with DNA ligase and the product used to transform *E. coli* DH5 cells (Bethesda Research Laboratories). Mutation recovery was improved by selection against the parental strand using *E. coli* host CAG629 (dut-ung-BioRad) to prepare the single strand template. In presence of M13K07 helper phage (Pharmacia), one strand of the plasmid DNA was copied, packaged into phage capsids and secreted into the supernatant, permitting isolation of the single strand DNA. Because this strand contained uracil, it was degraded when the mutagenesis reaction mix is used to transform *E. coli* DH5($Dut^{+}Ung^{+}$), leaving the mutagenized strand as template for replication. The modified TAP sequences were transferred as a *BamH*I cassette to the pKH4α2 vector.

This method is employed using the expression vector as the template for the mutagenesis reaction.

<u>Vector Construction</u>

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are

generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g. New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution. Typically, an excess of restriction enzyme is used to ensure complete digestion of the DNA substrate. Incubation times of about 1 to 2 hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by running over a Sephadex® G-50 spin column. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is formed in *Methods in Enzymology* (1980) : 499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris, pH 7.6, 50 mM NaCl, 6mM $MgCl_2$, 6mM DTT and 5-10 μM dNTPs. The Klenow fragment fills in 5' overhangs but removes protruding 3' single strands, even in the presence of the four dNTPs. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated followed by running over a Sephadex® G-50 spin column. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

As mentioned above, oligonucleotides may be prepared by the triester method of Matteucci, *et al.*(*J. Am. Chem. Soc.* (1981) *103*:3185) or using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labelling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 0.1 nmol substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7

pmoles ^{32}P-ATP(2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Ligations are performed in 15-30 µl volumes under the following standard conditions and temperatures: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 µM total ends concentration.

In vector construction employing "vector fragments," the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal phosphatase (CIP) in order to remove the 5' phosphate and prevent self-ligation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{2+} using about 1 unit of BAP per µg of vector at 60°C for about 1 hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated and desalted by application to a Sephadex® G-50 spin column. Alternatively, self-ligation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Site specific primer directed mutagenesis is conducted using a primer synthetic oligonucleotide complementary to a single stranded plasmid or phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the

original sequence. The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction were confirmed using Sequenase™ (U.S. Biochemical Corp.). Sequences can also be confirmed by first transforming *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D.B., *et al.*, *Proc. Natl. Acad. Sci. USA* (1969) *62*:1159, optionally following chloramphenicol amplification (Clewell, D.B., *J. Bacteriol.* (1972) *110*:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA* (1977) *74*:5463 as further described by Messing, *et al.*, *Nucleic Acids Res.* (1981) 9:309, or by the method of Maxam, *et al.*, *Methods in Enzymology* (1980) *65*:499.

Transformation

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., *Proc. Natl. Acad. Sci. USA* (1972) 69:2110, or the RbCl method described in Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual" (1982) Cold Spring Harbor Press, p.254 is used for procaryotes or other cells which contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens* (Shaw, C.H., *et al.*, *Gene* (1983) *23*:315) is used for certain plant cells. For mammalian cells without

such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology* (1978) *52*:546 is preferred. Transformations into yeast are carried out according to the method of Van Solingen, P., *et al.*, *J. Bacter*. (1977) *130*:946 and Hsiao, C.L. *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) *76*:3829.

Wild Type Recombinant TAP

European Publication 419099, Example 4, describes a procedure for expressing wild type recombinant TAP in yeast which involves synthesizing and constructing a recombinant gene encoding wild type TAP (described therein as "TAP-1") based on the amino acid sequence of wild type TAP. The synthetic gene was inserted into a yeast expression vector that allows for secretory expression. Yeast cells were transformed with the vector containing the synthetic gene. The described synthetic gene was inserted into pKH4 to form pKH4•TAP.

pKH4

Construction of pKH4 is described in Schultz, *et al.*, *Gene 61* (1987) 123-133, which is incorporated by reference. The plasmid pJC197 (Schultz *et al. Gene* 54 (1987) 113-123) is an *E. coli* - *S. cerevisiae* shuttle vector which contains a unique *BamH*I cloning site between the yeast *MFαl* pre-pro leader and transcriptional terminator, originally derived in Kurjan and Herskowitz (1982) ibid. pJC197 was digested with *EcoR*I + *Pst*I, and the 0.7-kb *Pst*I-*EcoR*I fragment containing a portion of the *MFαl* pre-pro-leader, a three-frame translational terminator, and *MFal* transcriptional terminator, was gel-purified. *GAL10p* was isolated from YEP51 by digestion with *Sau*3A, flush-ending with *Pol*Ik, ligating with octameric *BamH*I linkers, and digestion with *Sal*I.

The resulting 0.5-kb *BamH*I-*Sal*I fragment bearing *GAL10p* was gel purified and ligated to a 35-bp *Sal*I-*Pst*I synthetic oligodeoxynucleotide adapter encoding the first 11 bp of the *MFαl* nontranslated leader plus the ATG and first 8 aa of the *MFαl* pre-pro-leader. The resulting 0.5-kb fragment was digested with *BamH*I, gel-

purified, and ligated together with the aforementioned 0.7-kb *Pst*I-*EcoR*I fragment plus the 4.0kb *EcoR*I- *BamH*I vector fragment derived from pBR322. The resulting plasmid, pKH207-1, contains *GAL10p* fused to the *MFαl* pre-pro-leader plus *BamH*I cloning site, translational termination codons, and *MFαl* transcriptional terminator. Upon digestion with *EcoR*I and partial digestion with *BamH*I, an expression cassette of *GAL10p* fused to the yeast *MFαl* pre-pro-leader, a unique *BamH*I cloning site, translational termination codons in all three reading frames, and *MFαl* transcriptional terminator sequence was inserted into the yeast shuttle vector pC1/1 (Rosenberg *et al. Nature* 312 (1984) 77-80) which contains the yeast 2μ DNA sequence for stable propagation of the plasmid in yeast at high copy number, to form pKH4.

pKH4α2

A 213-bp *BamH*I-*Pst*I fragment encoding aa 9-79 of the ppL was prepared from the plasmid pa2 (Bayne *et al.*, *Gene* 66 (1988) 235-244). The plasmid pα2 contains a portion of the yeast *MFαl* pre-pro sequence (79aa) modified at codons 80 and 81 to create a *BamH*I site 6 aa upstream from the KEX2 processing site. The region corresponding to codon 9 (*Pst*I) of the ppL to the *BamH*I site of pKH4 was removed from pKH4 after digestion with *BamH*I followed by partial digestion with *Pst*I. The modified vector, pKH4α2 was prepared by replacement of this excised sequence with the *BamH*I-*Pst*I fragment from pa2. Plasmid pKH4α2 contains the yeast *GAL10* promoter, a portion of the *MFαl* pre-pro leader (79 aa), a three-frame translational terminator and *MFαl* transcriptional terminator, the yeast *LEU2* gene, yeast 2μ sequences, pBR322 derived sequences, including the Ap^R gene and the origin of DNA replication (ori).

Construction of pKH4 •TAP

Polymerase chain reaction resulted in a blunt end fragment which was regenerated in the usual fashion by digestion with *BamH*I. The correct fragment was obtained after electrophoresis on a 2%

agarose gel, excision of the band and electroelution. The purified fragment was ligated with the yeast expression vector pKH4α2 (Jacobson, M.A. *et al.* (1989) *Gene 85*: 513-518) that had been previously digested with *BamHI* and treated with calf alkaline phosphatase. The correct sequence of plasmid clones in the correct orientation was confirmed by DNA sequence analysis. Fusion products produced from pKH4•TAP are proteolytically processed by the Lys-Arg-cleaving endopeptidase (KEX2) encoded by the KEX2 gene and products are secreted into culture medium. KEX2 cleaves on the C-terminal side of Lys-Arg residues.

Transformation of DMY6

Diploid yeast strain DMY6 (Schultz, L.D. (1987) *Gene 61*: 123-133) was transformed with pKH4•TAP using standard protocols (Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA 75*: 1929-1933). One isolate was chosen and designated *S. cerevisiae* MY 2030 9718P281-3. The isolate was deposited with the American Type Culture Collection and identified ATCC No. 20984.

From plates containing yeast transformants, single colony isolates were obtained. These isolates were grown in 5X CM leu medium (0.85% yeast nitrogen base without amino acids and ammonium sulfate, 1% succinic acid, 0.6% NaOH, 0.5% ammonium sulfate, 0.03% isoleucine, 0.03% phenylalanine, 0.02% lysine, 0.02% tryptophan, 0.025% tyrosine, 0.02% adenine, 0.02% uracil, 0.01% arginine, 0.005% histidine, 0.01% methionine, 0.04% $FeCl_3.6H_20$, 0.03% $ZnSO_4$-$7H_2O$ and 4% glucose) at 30°C. Cells were grown by inoculating a frozen stock culture of 281-3 on leucine-minus agar media for 3 days at 28°C prior to the inoculation of 500 ml of selective medium (5 x CM Leu^-). The 500 ml seed culture was grown in a 2-1 Ehrlenmeyer flask for 16-18 hr at 28°C using a rotary shaker at 300 rpm prior to its inoculation into 2.5 1 of 5 x CM Leu^- broth containing 4% (w/v) galactose in a 1.5 1 Bio-Flo III fermentor (New Brunswick Scientific). The fermentor was operated at 28°C, 900 rpm, 2.5 1/min air for 110-120 hr.

All work was done at 2-8°C. Yeast cells were separated from broth (8 liters containing 1.69 gm. wild type recombinant TAP) of yeast culture (strain 281-3) secreting wild type recombinant TAP by diafiltering the liquid through a 500,000 MWCO hollow fiber cartridge (Romicon, PM500, 5ft) using an Amicon DC10 unit. The clarified broth was then diafiltered through a 100,000 MWCO hollow fiber cartridge (Amicon, H5P100-43) until approximately 500 mL of liquid remained in the reservoir. The retained liquid was diafiltered with 8 liters of 20mM BIS-TRIS, pH 6.0. (Buffer A). The effluent of the 100,000 MWCO fibers was adjusted to pH 6.0 and diluted with 63 liters of cold buffer A. The diluted broth contained 1.67 gm of wild type recombinant TAP . The diluted fermentation broth was divided into two 40 liter portions and wach was pumped at 2 liters/hr onto separate columns of Q-Sepharose Fast Flow™ (Pharmacia: 14 cm x 10 cm I.D.) that had been equilibrated with buffer A. After all the diluted broth had been pumped onto the columns they were each washed with 8 liters of Buffer A to finish eluting unbound materials. The bound proteins were eluted with 16 liters linear gradient of 0-500 mM NaCl in Buffer A. Fractions of 400 mL were collected and monitored for A_{280} and inhibition of human Factor Xa. Recombinant wild type TAP eluted at 175 mM NaCl.

The fractions that contained wild type recombinant TAP were combined and diluted with four volumes of 50 mM sodium acetate, pH 4.0 (Buffer B) before being pumped at 2 liters /hr onto a column of S-Sepharose Fast Flow™ (Pharmacia; 24.5 cm x 7.5 cm I.D.) that had been equilibrated with Buffer B. When all the sample had been pumped onto the column it was washed with 8 liters of Buffer B and then the bound proteins were eluted with a 16 liters linear gradient of 0-400 mM NaCl in Buffer B. The fractions were monitored for A_{280} and human Factor Xa inhibitory activity and wild type recombinant TAP eluted at 190 mM NaCl. The amount of wild type recombinant TAP eluted from the column was 1.58 gm. The pooled fractions containing inhibitory activity were loaded directly onto a 4.5 x 30 cm prep-pak C_{18} HPLC column (Waters Associates) equilibrated with

aqueous 0.1% (v/v) CF_3COOH at 50 ml/min using a Delta-Prep preparative HPLC system (Waters Assoc.). Following application of the sample at the same flow rate the column was washed with aqueous 0.1% (v/v) CF_3COOH for 5 min at 100 ml/min prior to the development of a CH_3CN gradient in aqueous 0.1% (v/v) CF_3COOH from 0-40% (v/v) at 1%/min. The peak of absorbance at 280 nm which eluted at a CH_3CN concentration of approximately 31% (v/v) was collected by hand and dried by lyophilization. Gel filtration chromatography can be used as an alternative to reserve phase HPLC to prepare desalted recombinant peptide. For gel filtration, Sephadex™ G-10 (Pharmacia) was swollen in Milli-Q™ H_2O and packed into a 2.5 cm (I.D.) X 93 cm glass column and 90 mL of a solution of wild type recombinant TAP purified by S-Sepharose chromatography was chromatographed on it. Fractions of 12.5 mL were collected and monitored for A_{280} and conductivity. Protein eluted in the V_0 and was well separated from the salt. The recovery of wild type recombinant TAP was 100%. The material was dried by lyophilization. The purified recombinant polypeptide prepared by either method was found to be greater than 99% pure by analytical reverse phase C_{18} HPLC, quantitative amino acid analysis and automated amino-terminal sequence analysis. A typical specific content of the recombinant inhibitor was between 115 and 120 nmole/mg with a homogeneous amino-terminus beginning with tyrosine. The electrophoretic mobility of wild type recombinant TAP was identical to the native inhibitor as assessed sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. The Factor Xa inhibitory activity of the wild type recombinant TAP was similar to natural TAP with a K_i determined to be 0.2 nM.

EXAMPLE 1

In order to illustrate specific preferred embodiments of the invention in further detail, the following exemplary laboratory work was carried out. This work includes the construction of a chemically synthesized TAP gene from selected oligonucleotides and cloning of the

gene in a suitable plasmid vector. A mutant recombinant TAP gene (Sequence ID No. 1) was then constructed by digestion of the coding sequences with selected restriction enzymes to afford desired DNA fragments. The fragments were isolated and ligated with selected synthetic oligonucleotides and introduced into an appropriate plasmid vector for cloning and subsequent expression of a double mutant recombinant TAP having the protein sequence represented by $Trp^{1}Arg^{10}$-TAP (see Figure 1, and Sequence ID No. 2).

Construction of rTAP Double Mutant

To prepare the single-stranded DNA template for site-directed mutagenesis, the DTAP open reading frame isolated from the yeast expression vector pKH4•TAP (Neeper *et al., J. Biol. Chem.* 1990 265, 17746-17752) was inserted into the vector pKH4-3B (Carty *et al., Bitechnol. Lett.* 1990 12, 879-994) in the following manner, to generate the plasmid p3B-TAP.

The pKH4-3B vector was digested with *Sph*I and the 3'-extensions were made flush-ended with T4-DNA polymerase. Following digestion with *Bgl*II, the blunt-*Bgl*II pKH4-3B vector was agarose gel purified, The wild type TAP gene was amplified by polymerase chain reaction with DNA oligomers that incorporated a *BamH*I site downstream of the wild type TAP gene on the 3'-end and a 5'-blunt end beginning with the DNA sequence encoding the first amino acid of wild type TAP. The wild type TAP gene was agarose gel purified and ligated with the pKH4-3B vector to yield p3B-TAP.

The wild type TAP clone was used to transform *E. coli* strain CJ 236 [dut^-, ung^-, thi^-, rel A^-, pDJ105 (Cm^r)] to produce single-stranded DNA template in the presence of helper phage M13K07 (Bio-Rad). The single-stranded DNA served as the template for annealing of mutagenic primers. Site-directed mutation of TAP was performed by using the Muta-gene M13 in vitro mutagenesis kit (Bio-Rad) according to the Kunkel method. For the single mutant with one change at the tenth amino acid (aspartate) to arginine, designated D10R, the template was annealed with an oligonucleotide, 5'-GTC-GAT-CCA-GCG-ACG-

CGG-TTT, extended by T4 DNA polymerase, and ligated. The double strand DNA was used to transform *E. coli* strain DH5 (dut^{+}, ung^{+}) in which the mutant sequence was propagated due to selective degradation of the uracil-containing parental strand. The double mutant, designated Y1W/D10R, which has a second mutation at the first amino acid (tyrosine) to tryptophan was produced by polymerase chain reaction (PCR) using the newly generated D10R mutant DNA as template. A sense oligonucleotide, 5'-TGG-AAC-CGT-CTG-TGC-ATC, and a 3' antisense oligonucleotide, 5'-ACTGGATCCGAATTCAAGCTTA-GATGCAAGCGT, were annealed with the D10R template and amplified by polymerase chain reaction. The PCR product was digested with *BamH*I to generate a cohesive 3'-end and ligated into the Sph I (blunted by T4 DNA polymerase) and *Bgl*II sites of the vector pKH4-3B. The double mutant DNA was propagated in the *E. coli* strain DH5 similar to mutant D10R. The sequences of the double-stranded DNA of D10R and Y1W/D10R produced by DH5 were confirmed using Sequenase™ (U.S. Biochemical Corp.).

Expression Purification of Double Mutant Y1W/D10R

The double-stranded DNA of Y1W/D10R was used to transform the *Saccharomyces cerevisiae* strain DMY6 (Mata/a, ade 1, ura3-52, his3::GAL10p-GAL4-ura3, leu2-2, 112/leu2-04, cir°/cir°) according to the procedure of Hinnen *et al.* (*Proc. Natl Acad. Sci..USA* 1978 75, 1929-1933) to form *S. cerevisiae* strain DMY6(TAP3). Transformation was selected on leu^{-} minimal medium agar plates, and confirmed with colony hybridization using ^{32}P-labeled, random primed TAP gene probe. Positive yeast colonies were inoculated in leu^{-} minimal medium with 4% glucose at 30°C overnight to an OD600 = 18-20. Cell was pelleted by low speed centrifugation and dispensed in 100 mL of leu^{-} medium with 8% galactose to reach OD600 = 1. The galactose induction continued for 5 days at 30°C, and the resulting broth containing secreted rTAP mutant proteins was separated from the cells by centrifugation.

The collected supernatant was filtered using 0.8μ filter units (Nalgene) with a glass filter paper, and diluted with 4 volumes of 50 mM acetate buffer (pH 4.0). The combined solution was adjusted to pH 4.0 with glacial acetic acid, and loaded onto a column (2.5 x 6 cm) of SP-Sepharose fast flow resin (Pharmacia) that had been pre-equilibrated with the same buffer in a cold room. The column was washed with 150 mL of acetate buffer, and then eluted with a 200 mL linear gradient from 0 to 1.2 M sodium chloride in acetate buffer, and fractions of 4 mL were collected. The fractions were assayed for Factor Xa inhibitory activity, and fractions containing rTAP double mutant were pooled. The collected solution was concentrated to 5-10 mL using Amicon with a YM3 membrane. The final purification was completed using reverse phase-HPLC with a semiprep C-18 peptide column (Vydac). The HPLC column was eluted with 0.1% trifluoroacetic acid (TFA) at a flow rate of 2 mL/min for 5 minutes, then a linear gradient from 0 to 25% 0.1% TFA in acetonitrile in 15 minutes, followed by another linear gradient from 25 to 40% TFA in acetonitrile in 30 minutes. The eluant was monitored at 280 and 220 nm using a diode array UV/Visible detector. Fractions containing rTAP double mutant protein were collected, concentrated, and subjected to a second HPLC purification. The final purified rTAP mutant was lyophilized to dryness and dissolved in HPLC grade water. The structure of the double mutant Y1W/D10R was confirmed by amino acid sequencing analysis.

EXAMPLE 2

Enzyme Assays and Determination of Kinetic Constants

The concentration of Y1W/D10R was determined by absorbance at 280 nm using an extinction coefficient of 22.2 $mM^{-1}cm^{-1}$ calculated from the method of Gill *et al.* (*Anal. Biochem.* 1989, 182, 319-326) or quantitative amino acid analysis with norleucine as the internal standard. Human Factor Xa was activated from human Factor X (purchased from Haematologic Technologies Inc.) by Russell's viper

venom Factor X activator by the procedure developed by Bock *et al.* (*Arch. Biochem. Biophys.* 1989 *273*, 375-388). The concentration of Factor Xa was determined by absorbance at 280nm using an extinction coefficient of 1.16 $(mg/mL)^{-1}$ and molecular weight of 46,000 (DiScipio *et al.*, *Biochemistry* 1977 16, 698-706) and confirmed by active-site titration (Bock *et al.* 1989).

To determine inhibition constants for the double mutant Y1W/D10R and wild-type rTAP, 1.5 nM Xa was incubated at room temperature with 4 to 10 nM of double mutant or wild-type rTAP in an assay buffer (TSP buffer) containing 20 mM Tris-HC1, pH 7.4, 150 mM NaC1, and 0.1% polyethylene glycol 8,000 (PEG 8000) in a PEG 20,000-coated microtiter plate for 2 to 3 hours. The residual substrate hydrolysis activity due to free Factor Xa was measured with 200 µM spectrozyme Xa (American Diagnostica). The inhibition constants were calculated from known total Factor Xa concentration, known total rTAP concentrations and free Factor Xa concentrations that were derived by comparing the residue activity with control experiments without rTAP.

To determine the association rate constants of double mutant and wild-type rTAP, 10 nM Factor Xa was mixed with an equal volume of 100-200 nM rTAP and 60 µM t-Boc-Ile-Glu-Gly-Arg-7-amido-4-methylcoumarin (Sigma) in TSP buffer using a stopped-flow spectrofluorometer (Applied Photophysics, model DX17MW interfaced with an Archimedes 420/I computer) according to the procedure described by Jordan *et al.* (*Biochemistry* 1992 31, 5374-5380). The fluorescence (excited at 380 nm) was monitored using a 400 nm cut-off filter at 25°C. The observed pseudo-first order rate constants were derived from the best fit of the fluorescence traces to an exponential approach of the velocity to a final steady-state value. Association rate constants were calculated by dividing observed rate constants by the rTAP concentration.

Characterization of the Double Mutant Y1W/D10R on Factor Xa

Table I lists inhibition constants (K_i) for the action of the double mutant and wild-type rTAP on human Factor Xa. The inhibition

constant for wild-type rTAP is similar to the literature value (Jordan *et al.* 1992). The double mutant shows an enhanced inhibitory potency relative to that of wild-type rTAP. Interestingly, both wild-type and double mutant rTAP exhibited the same association rate constant, k_{on}, (Table II). Therefore the increased inhibition potency indicates a slower dissociation rate for the Y1W/D10R:Factor Xa complex.

Table I

Protein	K_i(pM)
Wild-type rTAP	280
Y1W rTAP	150
D10R rTAP	100
Y1W/D10RrTAP	20

Table II

Protein	$k_{on}(M^{-1}s^{-1})$
Wild-type	1.8×10^6
Y1W/D10R	1.8×10^6

Therapy

The mutant rTAP of the invention can be used to inhibit blood coagulation inhibition by suitable administration to a patient in need of such treatment. The amount of mutant rTAP which would normally be administered is primarily dependent upon the physical characteristics of the recipient and the severity of the condition. The amount to be administered must be an effective amount, that is, an amount which is medically beneficial but does not present toxic effects which overweigh the advantages which accompany its use. The preferable route of administration is parenteral, especially intravenous. Intravenous administration of the mutant rTAP in solution with normal physiologic saline is illustrative. Other suitable formulations of the

mutant rTAP in pharmaceutically acceptable diluents or carriers in therapeutic dosage form can be prepared by reference to general texts in the pharmaceutical field such as, for example, Remington's Pharmaceutical Sciences, 16th edition, 1980, Mack Publishing Co. (Easton, PA)

Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention and it is intended that all such other examples be included within the scope of the appended claims.

The protein inhibits coagulation by inhibiting the Factor Xa pathway. Factor Xa pathway inhibition is achieved by administering the protein, either by continuous intravenous administration or bolus administration, in combination with a suitable pharmaceutical composition carrier e.g. saline, at a suitable pH, e.g. 7.4, such that the composition achieves the desired effect of preventing Factor Xa from inducing formation of thrombin from prothrombin.

The proteins form pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulphuric and phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids which form suitable salts include the mono, di and tricarboxylic acids. Illustrative of such acids are, for example, acetic, glycolic, lactic, pyruvic, malonic, succinic, trifluroacetic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic, 2-phenoxybenzoic and sulfonic acids such as methane sulfonic acid and 2-hydroxyethane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. Illustratively, these salts include those of alkali metals, as for example, sodium and potassium; alkaline earth metals, such as calcium and magnesium; light metals of Group IIIA including aluminium; and organic primary, secondary and tertiary amines, as for example, trialkylamines, including triethylamine, procaine,

dibenzylamine, 1-ethenamine; N,N'-dibenzylethylenediamine, dihydroabietylamine, N-(lower)alkylpiperidine, and any other suitable amine.

The anticoagulant dose of the proteins is from 0.01 mg/kg to 50 mg/kg of patient body weight per day depending on, for example, the patient, and the severity of the thrombotic condition to be treated. The suitable dose for a particular patient can be readily determined. Preferably from 1 to 4 daily doses would be administered typically with from 0.70 mg to 3.5 g of active compound per dose. The concentration of the proteinaceous substance of this invention having Factor X_a inhibition activity required to inhibit Factor X_a when used to inhibit blood coagulation or Factor X_a in a medium such as stored blood can be readily determined by those skilled in the art.

Anticoagulant therapy is indicated for the treatment and prevention of a variety of thrombotic conditions, particularly coronary artery and cerebrovascular disease. Those experienced in this field are readily aware of the circumstances requiring anticoagulant therapy. The term "patient" used herein is taken to mean mammals such as primates, including humans, sheep, horses, cattle, pigs, dogs, cats, rats, and mice. Inhibition of Factor Xa is useful not only in the anticoagulant therapy of individuals having thrombotic conditions, but is useful whenever inhibition of blood coagulation is required such as to prevent coagulation of stored whole blood and to prevent coagulation in other biological samples for testing or storage. Thus, the proteinaceous substance of this invention having Factor Xa inhibition activity can be added to or contacted with any medium containing or suspected of containing Factor Xa and in which it is desired that blood coagulation be inhibited.

Although the proteinaceous substance of this invention having Factor Xa inhibition activity may survive passage through the gut following oral administration, applicants prefer non-oral administration, for example, subcutaneous, intravenous, intramuscular or intraperitoneal administration by depot injection, or by implant preparation.

For parenteral administration the proteinaceous substance of this invention having Factor Xa inhibition activity may be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water and oils with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. Illustrative of oils which can be employed in these preparations are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, ethanol and glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

The proteinaceous substance of this invention having Factor Xa inhibition activity can be administered in the form of a depot injection or implant preparation which may be formulated in such a manner as to permit a sustained release of the active ingredient. The active ingredient can be compressed into pellets or small cylinders and implanted subcutaneously or intramuscularly as depot injections or implants. Implants may employ inert materials such as biodegradable polymers or synthetic silicones, for example, Silastic, silicone rubber or other polymers manufactured by the Dow-Corning Corporation.

The protein may be used alone or in combination with other proteins. For example, mutant rTAP enhances the efficiency of thrombolytic agents and mediated thrombolytic reperfusion. Such thrombolytic agents include, for example, tissue plasminogen activator, streptokinase, urokinase, and anisoylated plasminogen-streptokinase activator complex. Anisoylated plasminogen-streptokinase activator complex is commercially available from SmithKline Beecham as Anistreplase®. Mutant rTAP may be administered first following thrombus formation, and the thromboltyic agent is administered thereafter.

Deposit

S. cerevisiae DMY6(TAP3) deposited with the American Type Culture Collection, Rockville, MD, USA, is designated ATCC 74194. The deposit was made December 1, 1992 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent United States patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention rights granted under the authority of any government in accordance with its patent laws.

Description of Sequence IDs 1-10

Table III describes the sequences listed on the following pages. The shorthand notation references changes at amino acid positions 1 and 10 of the wild type TAP sequence. The sequence of wild type TAP is illustrated in Figure 1, with the exception that Figure 1 shows tryptophan at position 1 (instead of wild type tyrosine) and arginine at position 10 (instead of wild type asparate). Wild type TAP is also described in European Publication 419099.

Table III

Sequence ID No.	Molecule Type	Identity
1	genomic DNA	DNA for Y1W/D10R
2	protein	$Trp^{1}Arg^{10}$ (also Y1W/D10R)
3	protein	$Trp^{1}His^{10}$
4	protein	$Trp^{1}Lys^{10}$
5	protein	$Phe^{1}Arg^{10}$
6	protein	$Phe^{1}Lys^{10}$
7	protein	$Phe^{1}His^{10}$
8	protein	$Xaa^{1}Arg^{10}$
9	protein	$Xaa^{1}Lys^{10}$
10	protein	$Xaa^{1}His^{10}$

Xaa, a nonnaturally occurring amino acid, represents β-naphthyl alanine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Mao, Shi-Shan
Shafer, Jules
Huang, Jin

(ii) TITLE OF INVENTION: Anticoagulant Proteins

(iii) NUMBER OF SEQUENCES: 10

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(E) COUNTRY: US
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 32,586
(C) REFERENCE/DOCKET NUMBER: 18902P

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(C) TELEX: 138825

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 193 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGGAACCGTC TGTGCATCAA ACCGCGTCGC TGGATCGACG AATGCGACTC CAACGAAGGT      60
GGTGAACGTG CTTACTTCCG TAACGGTAAA GGTGGTTGCG ACTCCTTCTG GATCTGCCCG     120
GAAGACCACA CCGGTGCTGA CTACTACTCC TCCTACCGTG ACTGCTTCAA CGCTTGCATC     180
TAAGCTTGAA TTC                                                        193
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Trp Asn Arg Leu Cys Ile Lys Pro Arg Arg Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Trp Asn Arg Leu Cys Ile Lys Pro Arg His Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Trp Asn Arg Leu Cys Ile Lys Pro Arg Lys Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Phe Asn Arg Leu Cys Ile Lys Pro Arg Arg Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Phe Asn Arg Leu Cys Ile Lys Pro Arg Lys Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Phe Asn Arg Leu Cys Ile Lys Pro Arg His Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Xaa Asn Arg Leu Cys Ile Lys Pro Arg Arg Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Xaa Asn Arg Leu Cys Ile Lys Pro Arg Lys Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Xaa Asn Arg Leu Cys Ile Lys Pro Arg His Trp Ile Asp Glu Cys Asp
1               5                   10                  15

Ser Asn Glu Gly Gly Glu Arg Ala Tyr Phe Arg Asn Gly Lys Gly Gly
            20                  25                  30

Cys Asp Ser Phe Trp Ile Cys Pro Glu Asp His Thr Gly Ala Asp Tyr
        35                  40                  45

Tyr Ser Ser Tyr Arg Asp Cys Phe Asn Ala Cys Ile
    50                  55                  60
```

WHAT IS CLAIMED IS:

1. A protein which inhibits coagulation Factor Xa having the amino acid sequence:

$$
\begin{array}{l}
\text{AA}^{1}\text{-Asn-Arg-Leu-Cys-Ile-Lys-Pro-Arg-AA}^{10}\text{-Trp-Ile-Asp-Glu-Cys-} \\
\quad 1 \qquad\qquad\qquad 5 \qquad\qquad\qquad\qquad 10 \qquad\qquad\qquad\qquad 15 \\[1em]
\text{Asp-Ser-Asn-Glu-Gly-Gly-Glu-Arg-Ala-Tyr-Phe-Arg-Asn-Gly-Lys-} \\
\qquad\qquad\qquad 20 \qquad\qquad\qquad\qquad 25 \qquad\qquad\qquad\qquad 30 \\[1em]
\text{Gly-Gly-Cys-Asp-Ser-Phe-Trp-Ile-Cys-Pro-Glu-Asp-His-Thr-Gly-} \\
\qquad\qquad\qquad 35 \qquad\qquad\qquad\qquad 40 \qquad\qquad\qquad\qquad 45 \\[1em]
\text{Ala-Asp-Tyr-Tyr-Ser-Ser-Tyr-Arg-Asp-Cys-Phe-Asn-Ala-Cys-Ile} \\
\qquad\qquad\qquad 50 \qquad\qquad\qquad\qquad 55 \qquad\qquad\qquad\qquad 60
\end{array}
$$

or conservative amino acid substitutions thereof, wherein:

> AA^{1} is a hydrophobic amino acid, and AA^{10} is a hydrophilic amino acid,

or naturally occurring mutations, allelic variants, and micro-heterogeneous forms which conserve activity, provided that when AA^{1} is tyrosine, AA^{10} is not aspartate.

2. A protein of Claim 1, wherein AA^{1} has an aromatic side chain.

3. A protein of Claim 1, wherein AA^{10} has a polar side chain.

4. A protein of Claim 1, wherein AA^{1} has an aromatic side chain and AA^{10} has a polar side chain.

5. A protein of Claim 4, wherein AA^1 is selected from the group of amino acids consisting of tyrosine, tryptophan, phenylalanine, or β-naphthylalanine, and AA^{10} is selected from the group of amino acids consisting of aspartate, arginine, lysine, and histidine, provided that when AA^1 is tyrosine, AA^{10} is not aspartate.

6. A protein of Claim 5, wherein AA^1 is tryptophan and AA^{10} is aspartate.

7. A protein of Claim 5, wherein AA^1 is tyrosine and AA^{10} is arginine.

8. A protein of Claim 5, wherein AA^1 is tryptophan and AA^{10} is arginine.

9. A protein of Claim 1 for use in inhibiting Factor Xa, inhibiting thrombin formation, inhibiting thrombus formation, and inhibiting embolus formation, in a mammal.

10. A composition for inhibiting Factor Xa in a mammal, comprising a protein of Claim 1 and a pharmaceutically acceptable carrier.

11. A composition for inhibiting thrombin formation in a mammal, comprising a protein of Claim 1 and a pharmaceutically acceptable carrier.

12. A composition for inhibiting embolus formation in a mammal, comprising a protein of Claim 1 and a pharmaceutically acceptable carrier.

13. A composition for inhibiting thrombus formation in a mammal, comprising a protein of Claim 1 and a pharmaceutically acceptable carrier.

14. A composition of Claim 13 further comprising an anticoagulant agent, an antiplatelet agent, or a thrombolytic agent.

15. A composition for inhibiting Factor Xa in a mammal, comprising a protein of Claim 8 and a pharmaceutically acceptable carrier.

16. A composition for inhibiting thrombin formation in a mammal, comprising a protein of Claim 8 and a pharmaceutically acceptable carrier.

17. A composition for inhibiting embolus formation in a mammal, comprising a protein of Claim 8 and a pharmaceutically acceptable carrier.

18. A composition for inhibiting thromus formation in a mammal, comprising a protein of Claim 8 and a pharmaceutically acceptable carrier.

19. A composition of Claim 18 further comprising an anticoagulant agent, an antiplatelet agent, or a thrombolytic agent.

20. A method for inhibiting Factor Xa in a mammal comprising administering to the mammal an effective amount of a composition of Claim 10.

21. A method for inhibiting thrombin formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 11.

22. A method for inhibiting embolus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 12.

23. A method for inhibiting thrombus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 13.

24. A method for inhibiting thrombus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 14.

25. A method for inhibiting Factor Xa in a mammal comprising administering to the mammal an effective amount of a composition of Claim 15.

26. A method for inhibiting thrombin formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 16.

27. A method for inhibiting embolus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 17.

28. A method for inhibiting thrombus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 18.

29. A method for inhibiting thrombus formation in a mammal comprising administering to the mammal an effective amount of a composition of Claim 19.

30. A method for achieving thrombolytic reperfusion following thrombus formation in a patient comprising administering a

protein of Claim 1 to inhibit Factor Xa and thereafter administering a thrombolytic agent.

31. A method of Claim 30, wherein the thrombolytic agent is selected from the group consisting of tissue plasminogen activator, streptokinase, urokinase, and anisoylated plasminogen-streptokinase activator complex

32. A gene having DNA coding for the protein of Claim 1.

33. A recombinant molecule comprising DNA coding for the protein of Claim 1 consisting essentially of a DNA sequence coding for expression in a host for a polypeptide which inhibits coagulation Factor Xa, the DNA sequence being operatively linked to an expression control sequence in the DNA molecule.

34. A method for producing a protein of Claim 1 which inhibits coagulation Factor Xa comprising the steps of:

a) culturing a molecular host transformed with a recombinant DNA molecule, said molecule comprising DNA coding for the protein of Claim 1, said DNA consisting essentially of a DNA sequence coding for expression in a host for a polypeptide which inhibits coagulation Factor Xa, the DNA sequence being operatively linked to an expression control sequence in the DNA molecule; and

b) collecting the protein.

35. A gene having DNA coding for the protein of Claim 8.

36. A recombinant molecule comprising DNA coding for the protein of Claim 8 consisting essentially of a DNA sequence coding for expression in a host for a polypeptide which inhibits coagulation Factor Xa, the DNA sequence being operatively linked to an expression control sequence in the DNA molecule.

37. A method for producing a protein of Claim 8 which inhibits coagulation Factor Xa comprising the steps of:

a) culturing a yeast cell molecular host transformed with a recombinant DNA molecule, said molecule comprising DNA coding for the protein of Claim 8, said DNA consisting essentially of a DNA sequence coding for expression in a host for a polypeptide which inhibits coagulation Factor Xa, the DNA sequence being operatively linked to an expression control sequence in the DNA molecule; and

b) collecting the protein.

38. A method of Claim 37 wherein the yeast cell molecular host is *S. cerevisiae* DMY6(TAP3) .

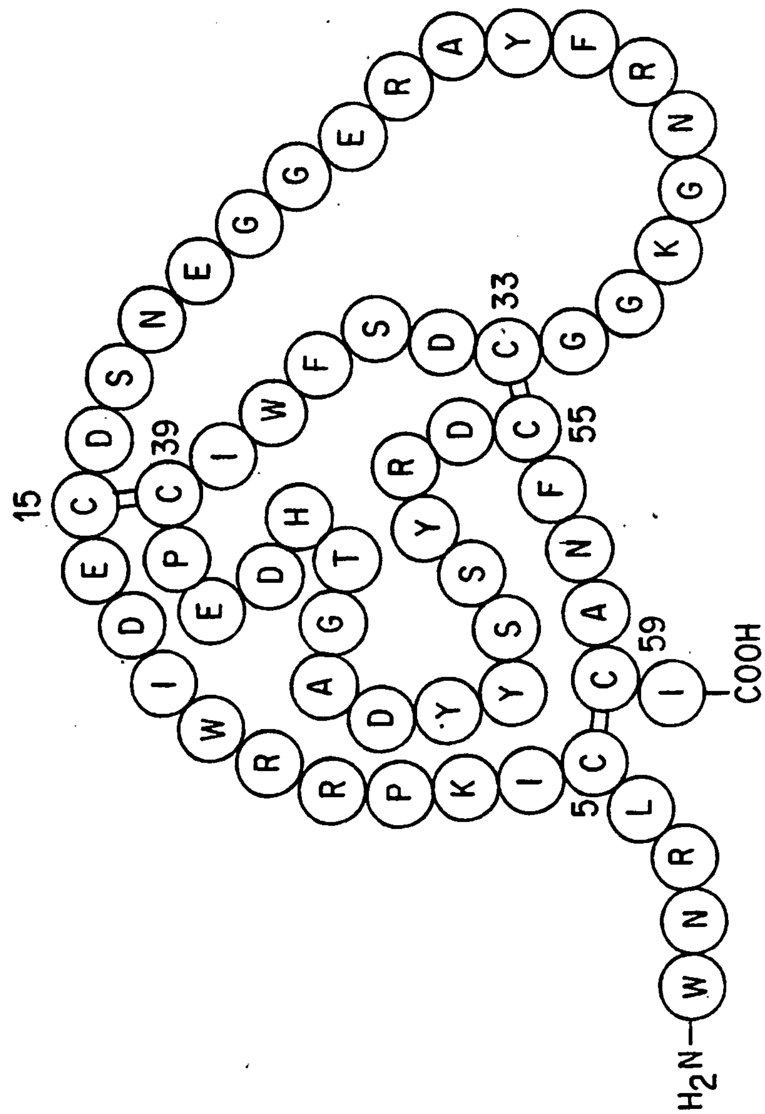

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/11154

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02; C07K 7/10, 15/08; C12N 15/04, 15/09, 15/15
US CL :435/69.2, 255, 320.1; 514/12; 530/324; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.2, 255, 320.1; 514/12; 530/324; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, PIR, SWISSPROT. search terms: sequence tick, anticoagulant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0,419,099 (Vlasuk et. al.) 27 March 1991. See the abstract, lines 10-13 of page 5, lines 12-15 of page 18 and pages 20 and 21.	1-38
Y	Journal of Molecular Biology, Volume 188, issued 1986, W. R. Taylor, "Identification of protein sequence homology by consensus template alignment", pages 233-258, see Table 1 on page 240.	1-38
Y	The Journal of Biological Chemistry, Volume 265, Number 29, issued October 1990, Neeper et. al., "Characterization of recombinant tick anticoagulant peptide", pages 17746-17752, see the abstract and pages 17748 and 17751.	1-38
Y,P	US, A, 5,189,019 (Palladino et. al.) 23 February 1993, see columns 15 and 16.	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1994	04 FEB 1994

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